Horizontal Ridge Augmentation

Michael M. Bornstein, Dieter D. Bosshardt, Thomas von Arx, and Daniel Buser

10.1 General Overview

10.1.1 Background

Clinical or experimental studies analyzing outcomes of horizontal (lateral) ridge augmentation can be divided into two categories: first, studies on the surgical augmentation procedure itself, in which the principal outcome parameter is the possibility to place a dental implant in an ideal position for prosthodontic rehabilitation without the need for additional grafting; and studies evaluating implant survival or success in horizontally augmented alveolar ridges according to predefined criteria. Techniques for horizontal ridge augmentation can be divided according to the type of grafting material used to augment or cover the surgical site (block or particulate/autogenous versus bone-substitute material/combinations of grafting material) or the type of membrane (resorbable or nonresorbable/natural versus synthetic material). A recent systematic review of the literature including only those studies with at least 10 or more patients and a minimum follow-up period of 12 months after loading of the inserted implants analyzed the outcomes of bone augmentation procedures in localized defects in the alveolar ridge (Jensen and Terheyden, 2009). The authors concluded that the most predictable horizontal ridge augmentation procedure included an autogenous block graft alone or in combination with a particulate bone graft or bone-substitute material, with or without the concomitant use of a resorbable membrane. In another recent systematic review applying similar inclusion criteria, the authors evaluated clinical outcomes of guided bone regeneration (GBR) procedures to correct dehiscence or fenestration type defects associated with implant placement (Chiapasco and Zaniboni, 2009). The authors found that it was difficult to draw significant conclusions with regard to any grafting material or membrane barrier for the treatment of

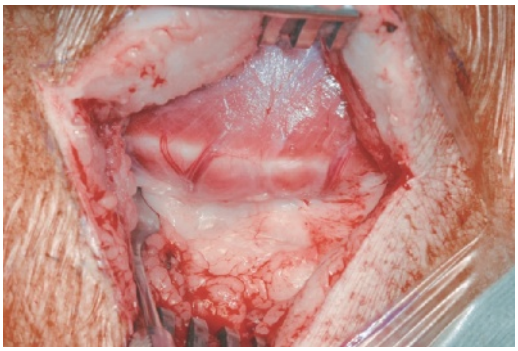

Fig 10-1 Through a subangular incision, the lateral portion of the mandibular body and ramus has been exposed to evaluate different grafting materials using minipigs.

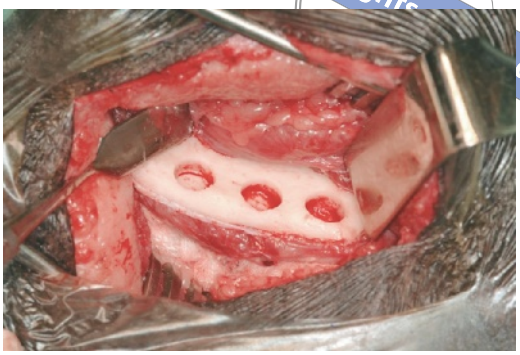

Fig 10-2 Three standardized intraosseous defects (9 mm in diameter/4 mm in depth) prepared at the area of the mandibular angle using a trephine and copious saline irrigation.

dehiscence/fenestration defects. Problems encountered were the limited sample sizes, the wide variety of grafting materials and membranes applied, either alone or in combination, and a paucity of randomized clinical trials.

10.1.2 Animal Models

Regarding the limited knowledge of GBR procedures and outcomes as derived from human clinical trials, the use of appropriate animal models for translational research in bone tissue engineering and regenerative medicine appears crucial. When choosing a species of animal for a particular study model or a specific research question, several factors should be considered, including: costs of acquiring and caring for the animals, ease of housing and availability, acceptability to society, biologic characteristics analogous to humans, and tolerance to surgery. Further, the lifespan of the species chosen should be suitable for the duration of the study, and the size of the animal must be appropriate for the surgical technique to be evaluated (Schimandle and Boden, 1994). In addition, regarding the research field of *horizontal ridge augmentation*, there are specific clinically related issues that further guide the decision making for an appropriate animal model (von Arx *et al.*, 2001a), including: surgical access to the area of augmentation (extraoral versus intraoral), type and size of bone defect (acute versus chronic, contained or transosseous), and defect localization (extraoral bone versus jawbone; ramus versus alveolar ridge). Taking into consideration all these factors, it is evident that within a specific field of research, no single animal model will be appropriate to study all purposes (Hazzard *et al.*, 1992).

In general, preclinical translational testing is ideally performed in large and skeletally mature animals, rather than in rodents or rabbits. Mimicking the underlying bone biology of the human is one of the principal goals of selecting a given animal (Schimandle and Boden, 1994; Liebschner, 2004; Egermann *et al.*, 2005). In rodents, the trabecular bone compartment is limited, even in metaphysic or long bones, and their skeleton continues to remodel throughout their lives at a faster rate than in humans. These are significant disadvantages when considering rodents as appropriate models of human bone biology. The dog, goat, sheep, and pig are the most utilized species when studying bone repair or bone regeneration (O'Loughlin *et al.*, 2008). Although nonhuman primates (NHPs) such as Rhesus macaque (Hanisch *et al.*, 2003) or baboons (Busenlechner *et al.*, 2005; Miranda *et al.*, 2005) have been used in experiments

Table 10-1 Overview of characteristics and key features of different animal species used for experimental research in the field of horizontal ridge augmentation

	Rat	Rabbit	Sheep	Pig	NHP	Dog
Different types (most common)	Norway rat (*Rattus norvegicus*), black rat (*Rattus rattus*), and albino strains	Californian, Florida White, and New Zealand White	Domestic sheep (*Ovis aries*)	Domestic pig (*Sus scrofa*)	Rhesus macaque (*Macaca mulatta*), baboon (*Papio anubis*)	Beagle, coonhound, mongrel
Adult weight	70–300 g	1.5–2.5 kg	>100 kg	50–350 kg	5–20 kg	<10 kg–>30 kg
Lifespan	4 years	9 years	10–12 years	10 years	20–40 years	15 years
Skeletal maturity	Continuous growth	1 year	1.5 years	1–1.5 years	5–7 years	1–1.5 years
Suitability/ applicability/ commonness	++	+	+	++	++	+++

+ = least suitable/common; ++ = moderately suitable/common; +++ = most suitable/common.
NHP, nonhuman primate.
Source: Pearce *et al*. (2007); Muschler *et al*. (2010).

analyzing the outcome of horizontal augmentation procedures of alveolar bone, the use of NHPs adds substantially to the cost of research and is associated with some cultural and ethical concerns (Muschler *et al*., 2010).

The dog is one of the more frequently used large animal species in orthopedic and dental research (Pearce *et al*., 2007). However, there are increasing ethical issues related to the use of dogs in medical research as well due to their status as companion animals. Sheep and goats have been used more frequently for orthopedic research in the last two decades, but for goats this was mostly limited to studies on cartilage, meniscal, and ligamentous repair (Pearce *et al*., 2007). Like sheep, goats are considered food-producing animals and thus also have the advantage of less critical public perception when used for research than companion animals such as dogs. Pigs are reported as the subject of choice for a variety of research topics including studies of fractures on cartilage and bone (Pearce *et al*., 2007) and studies assessing new dental implant surfaces (Buser *et al*., 1991, 2004) or bone grafting materials (Fig 10-1 and Fig 10-2; Buser *et al*., 1998; Jensen *et al*., 2006, 2009). Table 10-1 gives an overview of the animal species used for GBR research in the dental field.

Regarding the specific topic of lateral/horizontal ridge augmentation using different bone fillers with or without barrier membrane application and with or without simultaneous or staged implant insertion, different animal species have been utilized including rats (Kostopoulos and Karring, 1994; Donos *et al*., 2002), monkeys (Fonseca *et al*., 1983), sheep (Ylinen *et al*., 1991), and pigs (Buser *et al*., 1999; Mai *et al*., 2008; Bornstein *et al*., 2009). Some of these animal models do not represent the typical clinical situation encountered in patients with chronic and sometimes large bone defects on the buccal aspect of the alveolar ridge. Furthermore, there is a lack of literature reporting on failures or problems of specific experimental approaches evaluating horizontal ridge augmentation. One of the few studies to do so was published by Olsen and co-workers (2004), in

which the authors reported complete failure of their experimental setup comparing block and particulated bone grafts for ridge augmentation in combination with immediate implant placement using an intraoral approach in minipigs.

In Olsen's study, standardized bone defects (10 mm × 10 mm × 30 mm) were prepared on each side of the mandible of eight minipigs. After a healing period of 3 months, the defects in four animals were augmented with iliac crest grafts as a block or particulated graft with simultaneous implant insertion. Clinical inspection was performed after 2 weeks, and complete exposure of grafts and implants was discovered. The surgical procedures were altered in the fifth animal, avoiding incision in the insertion area of the musculus depressor labii mandibularis and placing the graft closer to the first molar. But again, grafts and implants were exposed. Consequently, the study was discontinued and all eight animals were sacrificed. The authors mention that the reasons for exposure of grafts and implants could be due to inappropriate handling of the animals in the stable and/or problems with the surgical techniques causing decreased blood supply in the surgical site. The authors concluded that an intraoral surgical design as described above cannot be recommended.

Therefore, the following sections of this chapter will focus on the most widely used and best established animal species for GBR procedures, and especially horizontal ridge augmentation, in dental research, the dog. Treatment modalities for the canine model include acute (Schwarz *et al*., 2007) and chronic (von Arx *et al*., 2001b, 2002) types of defects with varying sizes and configurations, different bone fillers (autografts, allografts, xenografts, alloplasts, and combinations), and different membranes to cover the augmented sites (bioabsorbable versus nonresorbable) (Schwarz *et al*., 2008a).

10.2 The Canine Model for Experimental Research in the Field of Horizontal Ridge Augmentation

10.2.1 Aim of the Canine Model

The animal species chosen for an experimental study addressing a specific clinical question should ideally fulfill the following prerequisites:

- Performance of therapy in a way it will most probably be carried out in the clinical setting
- Choice of a location for therapy/surgery that will as closely as possibly match the location in a patient
- Use of clinical methods that are similar/identical to those in the clinic
- Use of an animal that is comparable with humans regarding metabolic and physiologic characteristics
- Use of materials (grafts/membranes/transplants, etc.) that are similar/identical to the future clinical products.

There is substantial literature demonstrating that the canine model is one of the preferred animals for research of bone regeneration regarding the rich background of experience, ease of housing, and accessibility (Muschler *et al*., 2010). Therefore, this chapter will focus on the canine model in a step-by-step description of the surgery to histomorphometric methods most often applied in the laboratory.

The different outcome parameters evaluated in experimental studies analyzing horizontal ridge augmentation procedures have already been mentioned in the first paragraph of Section 10.1.1: namely, studies in which the principal outcome parameter is the possibility to place a dental implant in an ideal position without the need for additional grafting; and studies that evaluate implant survival or success in horizontally augmented alveolar ridges. Furthermore, the different techniques for horizontal ridge augmentation can be

Table 10-2 Potential variables for experimental studies evaluating horizontal ridge augmentation

Characteristics of the dog	Type of defect	Type of filler material	Type of barrier membrane	Implant insertion
Beagle dog (small) or mongrel dog (large)	Chronic type of horizontal ridge defect: Defect has to be prepared during extraction of teeth (initial surgery)	Negative control: no filler applied, represents physiologic regeneration/ repair process	Negative control: no barrier membrane applied, represents physiologic regeneration/ repair process	Outcome 1: Surgical augmentation procedure itself, in which the principal outcome parameter is the possibility to place a dental implant in an ideal position for prosthodontic rehabilitation
		Positive control: Usually autogenous bone in particulate or block form		
	Acute type of horizontal ridge defect: Defect is prepared during second stage surgery, e.g., simultaneously with augmentation procedure	Test sites: any modification of the situation mentioned above (allograft/ xenograft/ alloplast combinations in block or particulate form)	Positive control: Usually an ePTFE membrane	
			Test sites: Any modification of the situation mentioned above (non-resorbable/ resorbable/ combinations)	Outcome 2: Studies that evaluate implant survival or success according to predefined criteria in horizontally augmented alveolar ridges → dental implant placed during second stage surgery
Age: around 1 year				
Easy handling and manageability				
Intraoral approach				
Mandible > maxilla				
Extraction of teeth before horizontal ridge augmentation				
Macro- and microstructure of bone, and bone remodeling moderately similar to humans				
Bone composition most similar				

divided according to the grafting material used to augment or cover the surgical site (block or particulate/autogenous versus bone-substitute material/combinations of grafting materials) or the type of membrane (resorbable or nonresorbable/natural versus synthetic material).

Table 10-2 summarizes potential variables regarding the choice of dog species, the type of defect, type of filler material, and type of barrier membrane used for experimental horizontal ridge augmentation.

10.2.2 Advantages and Disadvantages of the Dog Model

The dog is one of the most frequently used large animal species for musculoskeletal and dental research. Regarding the macrostructure of bone, there may be some discrepancy in size and shape of canine bone in comparison with human bone, depending on the size and breed of the dog. This is an important aspect, as there is a wide variation between different dog species, and care should be taken when comparing the outcome of experimental procedures performed in animals that are physically different, as with large mongrel and small Beagle dogs. It is also interesting to note that despite similarities in organic composition, canine bone has a significantly higher mineral density than human bone (Wang *et al*., 1998). In terms of bone density, the dog and the pig most closely represent the human situation (Aerssens *et al*., 1998). In addition, it must be acknowledged that the remodeling process in dogs is much quicker than in humans, by approximately three to five times (Pearce *et al*., 2007; Reinwald and Burr, 2008). Consequently, even if two defects of the alveolar process regenerated with horizontal ridge augmentation procedures, one in a dog and the other in a human, exhibit the same size, the regeneration process as a whole will take longer for humans. Therefore, the regeneration process after horizontal ridge augmentation is similar in dogs and humans, but the time interval in which it takes place is different. On the other hand, the faster wound healing process in the dog model allows completion of experimental studies in a shorter time period than would be possible when performing a similar study with humans.

10.2.3 Timing of an Experimental Study Evaluating Horizontal Ridge Augmentation

Experimental studies evaluating horizontal ridge augmentation can be subdivided into four phases (Fig 10-3):

1. Planning phase – writing of the protocol, briefing of the coinvestigators including the veterinarian, submission of study plan to the local animal ethical committee for approval, inspection of the animal research facilities, and writing applications to various institutions and foundations for funding the study, if needed.
2. Surgical phase – extraction of teeth in the canine model described above, defect creation with/without simultaneous implant placement, augmentative procedures, healing phase, and sacrifice.
3. Histologic processing phase – for light microscopy, scanning electron microscopy (SEM), back-scattered SEM (BSEM), and transmission electron microscopy (TEM), descriptive morphology, histochemical and immunohistochemical methods, and histomorphometry with/without the help of specialized software.
4. Statistical analysis and manuscript draft preparation phase – depending on the number of animals included in the study, on the healing phases analyzed, and on the histologic techniques applied, the timeframe for an experimental study from the initial planning stages to the finalized manuscript may range from less than 1 to several years.

10.2.4 Surgical Procedures: General Thoughts

Experimental studies should only be initiated after approval has been granted by the responsible animal ethical committee. Writing a protocol for the committee should not only be regarded as a necessary bureaucratic step before beginning the study, but as a chance to critically assess the methods and outcome parameters chosen. Furthermore, this preparation period allows the scientist to talk to the veterinarian in charge about the study to choose adequate medication, diet, and follow-up visits, to inspect the housing facilities of the animals, and to check that after sacrifice, the necessary steps for the histologic processing

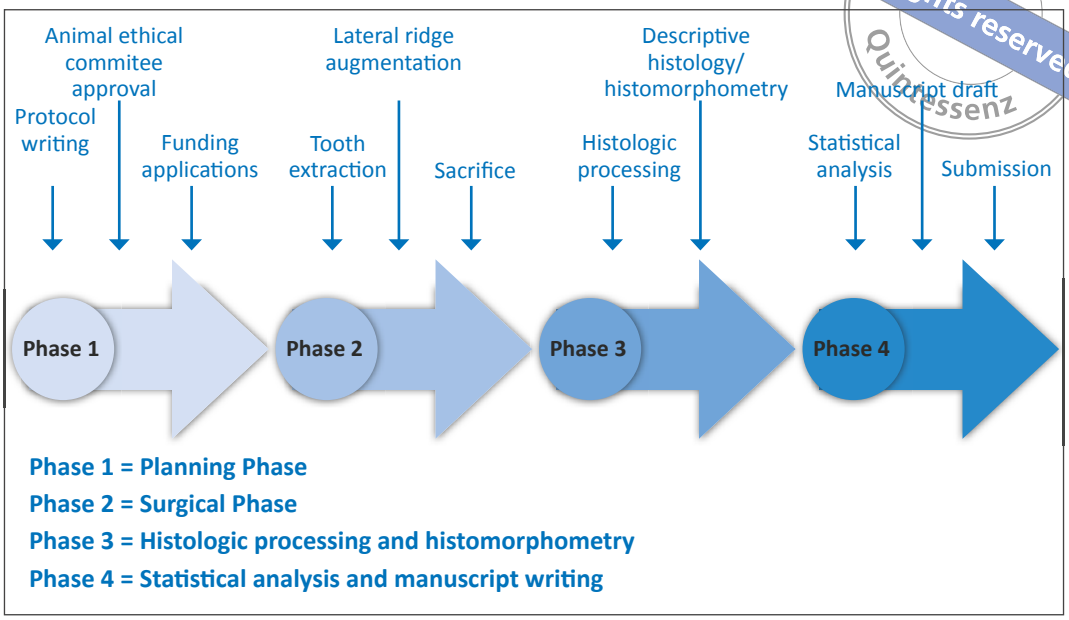

Fig 10-3 The four phases of an experimental project evaluating lateral/horizontal ridge augmentation in the canine model.

and analysis are clear to all involved investigators. Usually, all surgeries are performed under general anesthesia in an operating room under aseptic conditions. For horizontal ridge augmentation procedures in the dog model, usually two surgical procedures are necessary: (1) extraction of teeth (generally all premolars, and the first mandibular molar); and (2) horizontal ridge augmentation using one of the approaches specified in Table 10-2.

10.2.5 Preparation of the Animals

Dogs to be included in the study are selected in accordance to their general health, age, and weight. A certified veterinarian should examine the animals, and be responsible for their systemic health during the entire study. Animals that are approximately 1 year of age are preferred.

A typical setup for general anesthesia of a Beagle dog would be as follows (Bornstein *et al*., 2007): after intramuscular injection of atropine (0.05 mg/kg), anesthesia is induced by intramuscular administration of tiletamine-zolazepam (5 to 10 mg/kg), followed by a slow intravenous administration of sodium thiopental (10 to 15 mg/kg). Anesthesia is maintained with inhalation of an O_2/N_2O isoflurane (0.5% to 4%) mixture. Additionally, local anesthesia is achieved by buccal and lingual infiltration with 2% lidocaine combined with 1/100,000 epinephrine (adrenaline).

10.2.6 Detailed Surgical Methodology Including Sacrifice

Surgery 1 (tooth extraction)

This step prepares the alveolar crest for the experimental setup to be tested by removing the teeth in the area of interest. In general, sulcular incisions are made, with subsequent reflection of full mucoperiosteal flaps in the mandible from the canine to the second molar for better access and visualization of the teeth (Fig 10-4a). All premolars (PM1–PM4) and the first molar (M1) are then removed (Fig 10-4b). Prior to removal, all two-rooted teeth are sec-

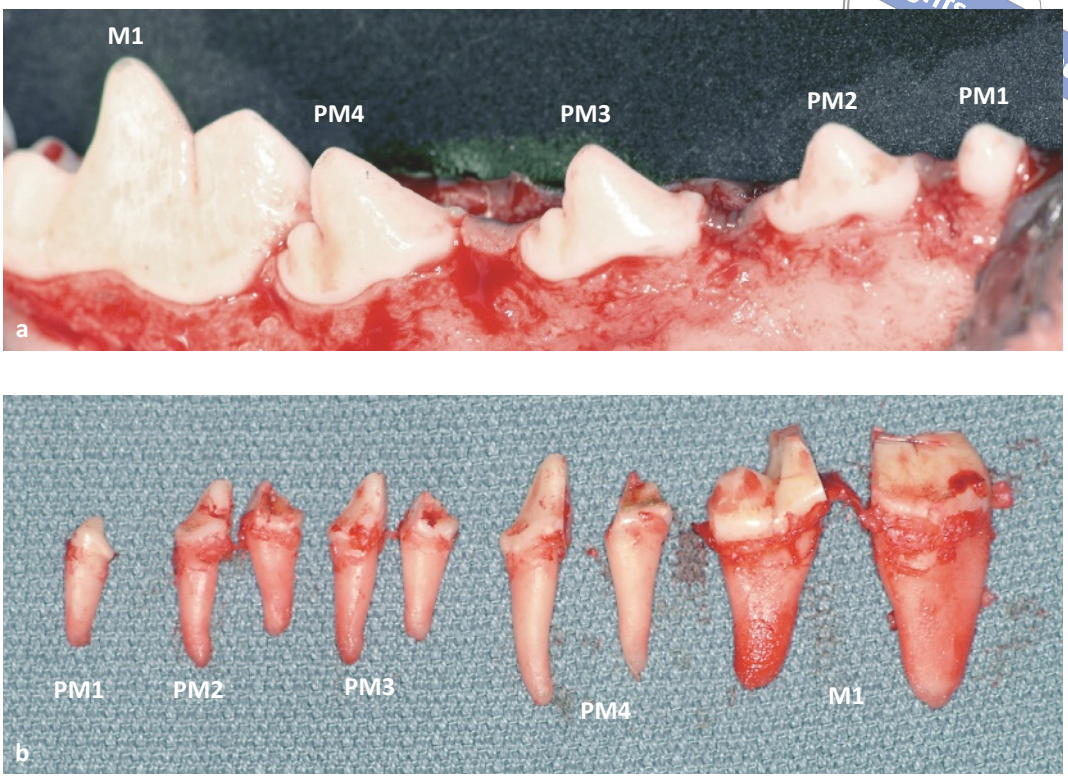

Fig 10-4 (**a**) Right mandible of a mongrel dog after preparation of lingual and buccal mucoperiosteal flaps from the first premolar (PM1) to the first molar (M1). Thus, access for extraction of the premolars (PM1 to PM4) and the first molar (M1) is simplified. (**b**) The extraction of the premolars (PM1 to PM4) and first molar (M1) should be done with great care not to fracture any root tips. Therefore, the two-rooted teeth (PM2, PM3, PM4, and M1) are sectioned using a separating disk before root extraction.

tioned with a separating disk, to ease root extraction. All drilling should be done under sterile saline irrigation. Finally, the flaps are closely approximated with interrupted sutures. Suture removal is generally done 1 to 2 weeks postoperatively. As modifications to this study design, defects of the alveolar ridge, ideally buccal defects, can be created at this stage (Figs 10-5a and 10-5b). This is always indicated when a chronic-type of defect is indicated for the study (von Arx *et al*., 2001a,b; Araújo *et al*., 2003; Schwarz *et al*., 2008b, 2009).

Surgery 2 (lateral/horizontal ridge augmentation) Generally, the alveolar crests and/or defect sites are re-entered for lateral ridge augmentation 2 to 3 months after the first surgery (von Arx *et al*., 2001a,b, 2002; Araújo *et al*., 2003; Bornstein *et al*., 2007; Schwarz *et al*., 2008b, 2009). For the second intervention, midcrestal incisions are made from the canines to the second molars in the mandible, and full-thickness mucoperiosteal flaps are elevated on the buccal and lingual sides. As a variation of this procedure, especially if only two different time points will be evaluated, defects could be prepared only on one mandibular side for a specific time point (Bornstein *et al*., 2007). Using this

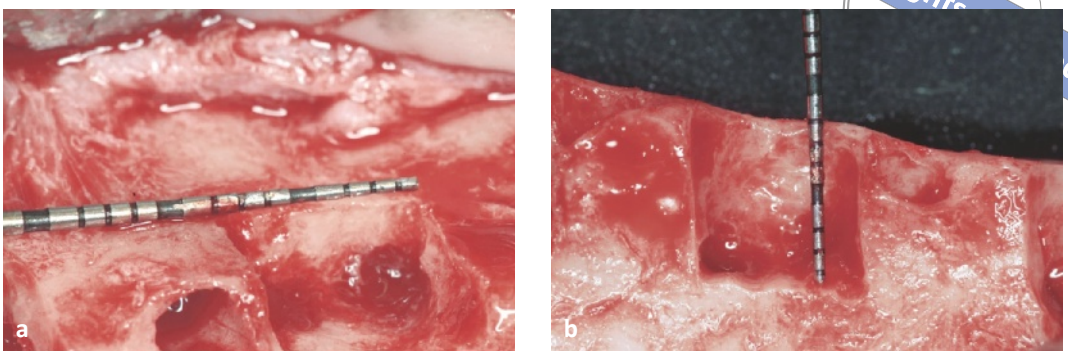

Fig 10-5 (**a**) After removal of the premolars and first molar in the right mandible of mongrel dogs, standardized defects on the buccal bone wall are created. For this experimental set-up, the mesiodistal width measures 9 mm (same canine mandible as in Fig 10-4a). (**b**) In the same canine mandible as in Fig 10-4a, the height from the crestal bone to the bottom of the defect measures 9 mm.

approach, each canine mandible comprises two different time points, and thus minimizes potential confounders (time or animal related). Nevertheless, randomization should be planned as regards location and different treatment options to be evaluated, when performing horizontal ridge augmentation procedures. If defects were not created during surgery 1 (= chronic-type defect), they are made at this stage in the edentulous alveolar mandibular area using rotary and hand instruments and chilled sterile saline irrigation (= acute-type defect). After defect creation in the alveolar ridge, horizontal ridge augmentation itself is performed. Many different variables exist for this procedure, ranging from autogenous bone blocks with varying sizes and configurations, particulate autografts, allografts, xenografts, alloplasts, and combinations thereof. Furthermore the defects can be covered without or with different membranes ranging from bioabsorbable to nonresorbable, and from collagen to synthetic polymers (Schwarz *et al*., 2008a; Fig 10-6). As a rule, the defects and augmented sites should ideally be measured with a periodontal probe. A further modification of this approach includes the simultaneous placement of dental implants, and the coverage/horizontal augmentation of any dehiscence

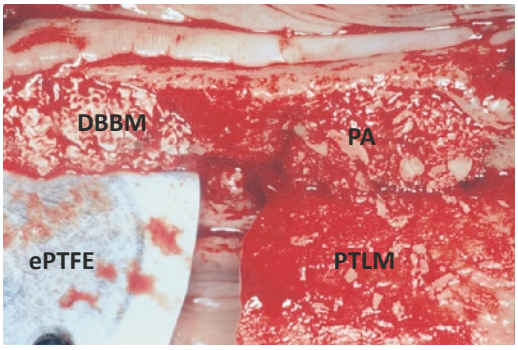

Fig 10-6 Two standardized lateral bone defects created in the mandible are augmented with bone graft material (PA, particulate autograft; DBBM, deproteinized bovine bone mineral) following stabilization of the membranes (bioresorbable prototype trilayer membrane [PTLM]; nonresorbable expanded polyfluorethylene [ePTFE]) at the lower buccal aspect with titanium screws.

defects (Fig 10-7). Before closure of the wound, the surgical site should be thoroughly irrigated with sterile saline to remove any residual debris. Following this, the wound margins are carefully approximated and closed with horizontal mattress and single interrupted sutures.

Sacrifice

Usually, dogs are sacrificed using an overdose of pentobarbital sodium 0.2 mL intravenously

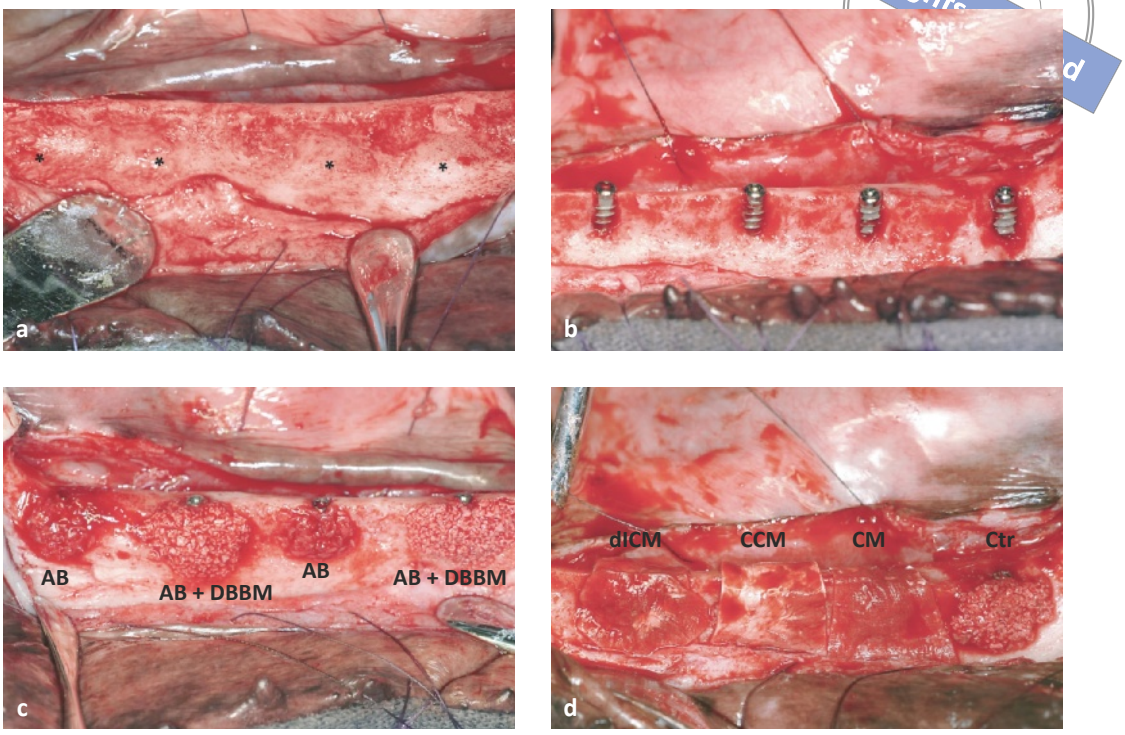

Fig 10-7 (**a**) Chronic-type defect 2 months after tooth extraction and creation of the defects (*) in the right canine mandible. (**b**) Insertion of four dental implants in the area of the chronic-type defects in the right canine mandible. All four implants exhibit pronounced buccal dehiscence defects. (**c**) The dehiscence defects are covered with either particulate autogenous bone grafts alone (AB) or in combination with particulate bovine bone mineral (AB + DBBM). (**d**) The grafted dehiscence defects are then covered with a commercially available non cross-linked collagen membrane (CM), a prototype cross-linked collagen membrane (CCM), and a double-layer of the non cross-linked collagen membrane (dlCM). Here, the control defect without membrane coverage (Ctr) is grafted with autogenous particulate bone in combination with particulate bovine bone mineral (AB + DBBM).

(65 mg/kg). There are also methods reporting perfusion with a fixative (formaldehyde-glutaraldehyde; Karnowsky, 1965), although the respective animals should be sedated before injection. Subsequently, the mandibles are resected en bloc, including the covering soft tissues, using an oscillating autopsy saw (Fig 10-8). The recovered specimens should be immediately immersed in a solution of formaldehyde (4%) combined with 1% calcium chloride prior to histologic preparation (alternative: neutral buffered 10% formalin solution).

10.2.7 Postoperative Care

After tooth extraction in the mandible (with or without simultaneous defect creation), animals normally receive an antimicrobial prophylaxis (for example: combination of spiramycin 750,000 IU and metronidazole 125 mg per day per os for at least 7 days), and an anti-inflammatory agent (for example: carprofen 50 mg per os and per day for three days). Additionally, animals receive a subcutaneous injection of an analgesic (for example: butorphanol). For suture removal under intravenous sedation, the following medications are used: atropine (0.05 mg/kg intramuscular) and tiletamine-zolazepam (5 mg/kg intra-

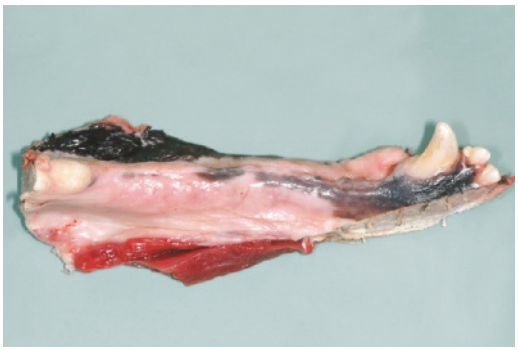

Fig 10-8 En-bloc-resected canine mandible, including the covering soft tissues, using an oscillating autopsy saw.

Fig 10-9 Micro-CT image of a central bone core retrieved during implant bed preparation.

muscular). A soft diet is generally maintained throughout the study, and the dogs should be regularly checked when in their cages to prevent them chewing on the bars or other bulky material with their operated mandibles.

10.2.8 Histologic Processing and Endpoint Measurements

Microscopic methods used to evaluate outcomes, efficacy, and quality of augmentative procedures in experimental studies include various techniques such as micro-computed tomography (micro-CT), light microscopy, scanning electron microscopy (SEM), backscattered electron microscopy (BSEM), transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM), and standard radiography, all of which provide excellent and useful information (Boyde *et al*., 1995).

Micro-CT

Following the fixation period, grafted regions of the retrieved specimens can be quantified via micro-CT analysis (Maréchal *et al*., 2005; Kon *et al*., 2009; Fig 10-9). Possible measurements include: total volume of newly formed bone (TBV; usually in cubic millimeters) and gain in bone height (BH). These measurements are based on standard two-dimensional image analysis that is further processed using stereologic methods (Saffarzadeh *et al*., 2009). TBV is acquired by the radiopaque voxels observed in the region of interest; the BH can be evaluated from the distance between the basal host (original) bone and the highest point of the regenerated bone.

Histologic Processing

Without applying the perfusion technique, the retrieved specimens are ready to be prepared for further histologic processing and analysis after a period of approximately 2 weeks. There are several procedures available for histologic processing, but one widely accepted method, and also the method favored by the authors of this chapter, is the histologic processing as described by Schenk and co-workers (1984). According to this procedure, the fixated block specimens are dehydrated and embedded in methylmethacrylate. The specimens are usually cut in a buccolingual direction in the regions of the defects. If dental implants are present, they should be cut parallel to their axis, resulting in two to three approximately 500 µm thick undecalcified sections per implant (with an implant

Fig 10-10 The ground sections are glued on a Plexiglas slab and ground to a final thickness of 80 to 100 µm.

diameter between 4 and 5 mm). Subsequently, the sections are glued to opaque Plexiglas slabs with acrylic cement, ground to a final thickness of approximately 80 µm (Fig 10-10), and stained superficially with toluidine blue alone or toluidine blue followed by basic fuchsin. Ideally, one should analyze as many coronal sections per defect as possible for descriptive histology and histomorphometry. However, implant diameter and technical issues such as tissue processing and cutting/grinding may reduce the number of sections available or suitable for analyses. If only one section is analyzed, it should comprise the most central coronal section.

Equipment for Histomorphometry

All measurements should be performed with a photomicroscope (color charge-coupled device camera mounted on a binocular light microscope) by an experienced examiner. Ideally, the observer should also be masked for the specific experimental condition(s). The digital images using different magnifications (between ×100 and ×200) can be evaluated using specific software programs. As an alternative, analysis can also be performed conventionally, for example by using a superimposed grid for point counting with standardized image magnifications. In fact, observation directly in the microscope offers significant advantages including focusing during observation and distinction between different tissues or maturation stages in critical situations. Thus, conventional stereologic methods are often preferred over computer-based analyses.

Histomorphometric Measurements using Light Microscopy

There are numerous possible histomorphometric measurements, and the most common parameter to be measured is the calculation of a specific area (in square millimeters) of interest. As landmarks, the coronal extension of the bone crest adjacent to the defect area, and the bottom of the bone defect usually are defined (Fig 10-11). Using these landmarks, additional measurements include: the total area of the membrane-covered compartment, the proportion of the different tissues found in the regenerated area such as bone matrix/newly formed bone, soft tissue and residual graft/filler material (expressed for example as a percentage of the regenerated area; Bornstein *et al*., 2007; Jensen *et al*., 2009), the mineralized bone to osteoid ratio, the bone-to-filler contact (to assess osteoconduction), and the bone-to-implant contact, when dental implants were inserted. Bone-to-filler and bone-to-implant contacts can be analyzed directly under the microscope using a square grid (Buser *et al*., 2004; Jensen *et al*., 2009), or by calculating interface contact lengths between bone and implant–bone filler surface using a software package (Bornstein *et al*., 2008). There are further selected light microscopy techniques to evaluate specific questions in experimental studies: the use of polarizing light microscopes (Fig 10-12; Saffarzadeh *et al*., 2009), fluorescence microscopy using different labeling techniques/dyes to visualize and quantify bone formation (for example with calcein blue, xylenol orange, calcein, or alizarin complexone; Fig 10-13) that were injected at different stages of the experiment (Aida *et al*., 2003; Katsaros *et*

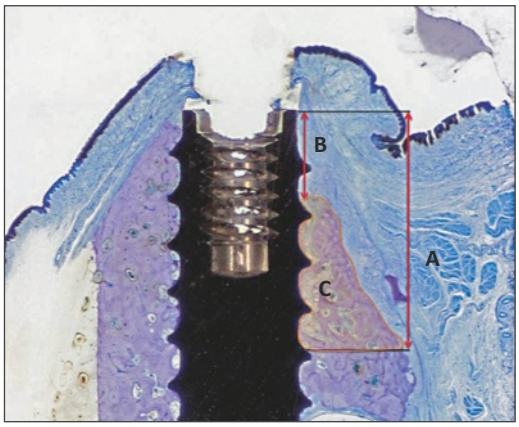

Fig 10-11 Histomorphometric measurement of a dental implant inserted in a canine mandible with a buccal dehiscence defect augmented with autogenous bone and covered with a cross-linked collagen membrane. A: distance from the bottom of the bone defect to the implant shoulder. B: distance from the first bone-to-implant contact to the implant shoulder. C: the total area of the membrane-covered regenerate.

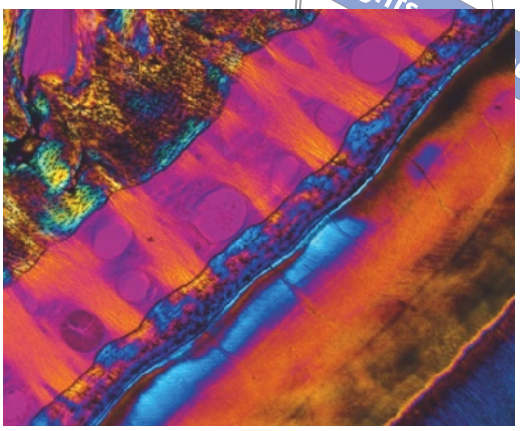

Fig 10-12 Ground section viewed under polarized light showing dentin, enamel, root cementum, periodontal ligament, and alveolar bone. This technique is particularly useful to illustrate the orientation of collagen fibers.

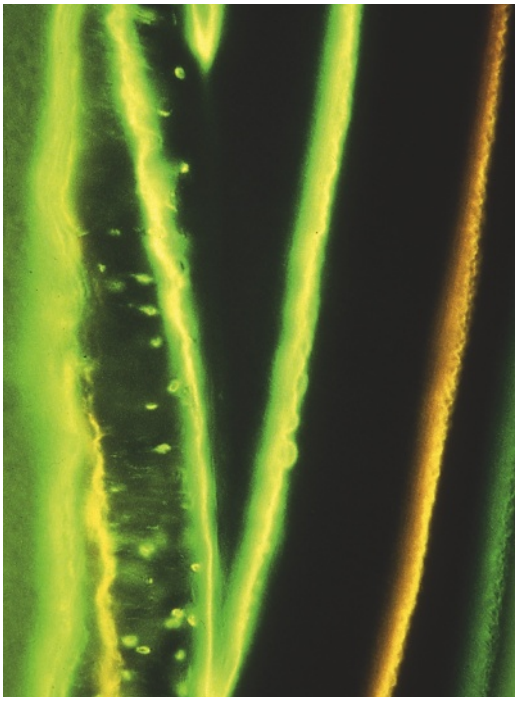

Fig 10-13 Fluorescent lines in cementum and dentin viewed in the fluorescence microscope. The animal received sequential injections of calcein (green lines) and xylenol orange (orange lines). The two fluorochromes bind to sites of ongoing mineralization and produce clear fluorescence lines.

al., 2006), bright field microscopy (Hwang *et al*., 2000), and phase contrast (Dereka *et al*., 2006).

For descriptive purposes, osteoclast-like cells can be stained histochemically by evaluating the activity of tartrate-resistant acid phosphatase (TRAP) in multinucleated giant cells with azo staining using naphtol AS-TR phosphate coupled with fast red violet TR salt (Jensen *et al*., 2009; Fig 10-14). Additionally, immunohistochemical labeling of the specimens allows for an analysis of selected antigen reactivity. For example, osteocalcin, a non-collagenous protein, which is predominantly synthesized by osteoblasts, odontoblasts, and hypertrophic chondrocytes, can be visualized in the tissues indicating maturation of regenerated bone areas by highlighting osteoblastic differentiation (Schwarz *et al*., 2007, 2008a,b). Another example of immunohistochemical analysis is the use of monoclonal antibodies to transglutaminase II (TG) (Schwarz *et al*., 2008a, 2009). As the organization of the wound area by proliferating blood vessels is considered to be of crucial importance for the process of GBR, angiogenesis can be investigated by

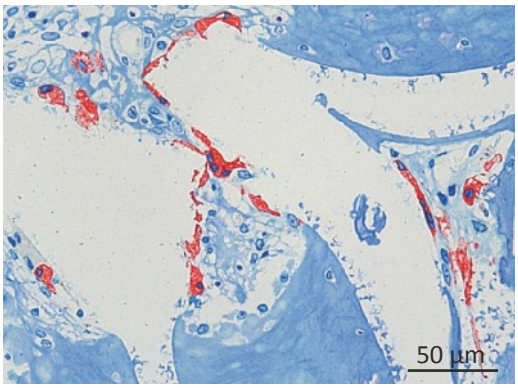

Fig 10-14 TRAP staining in a 1 µm thick section from a biopsy retrieved from a site augmented with a bone substitute material. The cytoplasm of TRAP-positive multinucleated cells, which are located at the biomaterial-soft tissue interface, stands out due to its dark red staining.

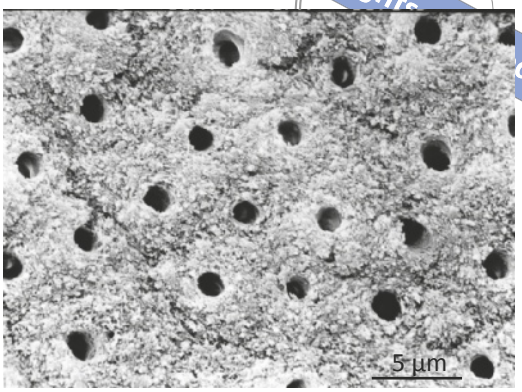

Fig 10-15 SEM view of the mineralized dentin after removal of the soft tissue of the dental pulp. Note the regularly arranged dentinal tubules.

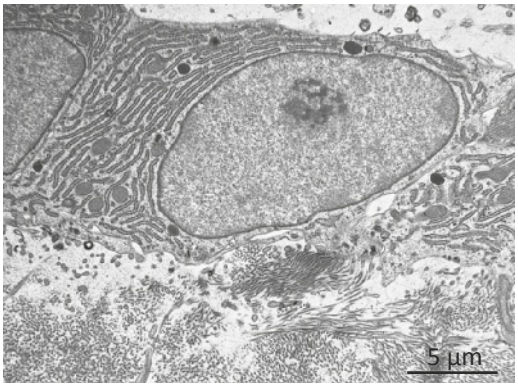

Fig 10-16 TEM illustrating a typical osteoblast adjacent to the osteoid matrix. Note the abundant rough endoplasmic reticulum in the cytoplasm.

labeling the tissues with antibodies to TG. Especially, the variations in transmembraneous angiogenesis between different types of barrier membranes can be studied using this technique (Schwarz *et al*. 2008a).

SEM/BSEM/TEM

SEM has been described in experimental studies after sputter-coating the fixated specimens with gold-palladium (de Kok *et al*., 2003; Saffarzadeh *et al*., 2009; Fig 10-15). With SEM imaging, bone filler particles with a low organic component and a relatively high atomic number of calcium and phosphate in hydroxyapatite crystals usually appear whitish-gray, whereas newly formed bone appears dark gray because of collagen, marrow, and fat components (Traini *et al*., 2008).

BSEM offers considerable insight into the mineralized tissues at the graft–bone and/or implant–bone interface. BSEM is particularly useful in distinguishing one material from another, since the yield of the collected backscattered electrons increases monotonically with the specimen's atomic number (Boyde and Jones, 1983; Nanci *et al*., 1990). A recent experimental study in the canine mandible used BSEM to analyze specimens in which bone was augmented both horizontally and vertically with a xenograft scaffold and recombinant human platelet-derived growth factor (rhPDGF-BB), with or without a resorbable collagen membrane (Rocchietta *et al*., 2007).

For TEM, ultrathin sections (ideally less than 100 nm in thickness) are collected on copper grids, stained with lead citrate and uranyl acetate, and examined under the microscope

(Orsini *et al*., 2006; Fig 10-16). Using TEM, Orsini and coworkers were able to observe and differentiate the following features: regions rich in osteoid matrix and spaces between the collagen fibrils; areas in which there was a rich-interlaced framework of collagen fibrils that started to present circumscribed mineralization foci; regions of woven bone; and zones of well-organized mature bone. A disadvantage of TEM is its limitations when a metal implant is present in the biopsy material.

A technique that is a little less common, but still is worth noting here is contact microradiography. This technique is especially suitable for descriptive analyses due to the good contrast seen, for example, between metal implants and surrounding bone, for different stages of bony maturation/mineralization, or between native/older and newly regenerated bone (Sawai *et al*., 1996; Tung *et al*., 2006; Chikazu *et al*., 2007).

10.2.9 Thoughts on the Statistical Analysis

Researchers evaluating horizontal ridge augmentation procedures and outcomes invest great effort in terms of time and money to ensure that the experiment addresses an important question in a biologically valid and meaningful way. Nevertheless, often little thought is given to ensure that the experimental data will be collected and analyzed in such a way as to provide a valid answer to the research question that was framed with great care (Stratton and Neil, 2005). It is important to remember that inappropriate analyses and/or erroneous interpretations can invalidate the meaning and impact of the data collected. A thorough planning of an experimental study (phase 1, see 10.2.3 and Chapter 4) should therefore already include an evaluation of the statistical methods to be applied to ensure that the study is likely to yield conclusive results. Furthermore, given the expensive nature of *in vivo* experiments and the ethical concerns involved, careful planning is mandatory to ensure that animals are used efficiently (Hanfelt, 1997).

An analysis to identify the statistical significance should prove that the difference between groups did not happen by chance alone. It should be emphasized here that there is never 100% certainty that chance did not play a role in the data collected, but we can calculate the probability that chance alone was not the dominating factor in the results. That probability is called the *P* value (Baumgardner, 1997; Whitley and Ball, 2002a). Another important variable is the confidence interval that indicates the likely range of values for a certain effect in the population studied. Confidence intervals and *P* values are both strongly dependent on the size of the study sample, with larger samples generally resulting in narrower confidence intervals and smaller *P* values.

Sample sizes for experimental studies using a canine model should ideally be kept to a minimum and therefore, they should be rationally planned and not arbitrarily chosen. This makes calculation of an appropriate sample size important, and more and more animal ethical committees as well as grant-giving bodies are requiring adequate sample size calculations to be provided at the initial stage of the project (Whitley and Ball, 2002b). Factors that affect sample size calculations are: a cutoff for statistical significance based on a defined *P* value; the size of the effect to be detected, with a small effect requiring larger samples; the statistical power of the study, e.g. the probability of correctly identifying a difference between groups in the study sample when one genuinely exists. It should be kept in mind that sample size calculations, when performed at the initial stages of an experimental study (phase 1), are by large dependent on estimates of effect, power, and significance. Thus, a range of values should be initially provided in order to give several suitable sample sizes rather than a single number. To help researchers with this crucial step, several computer programs are available for adequate sample size calculation.

By being aware of the importance of accurate sample size calculation, researchers can avoid performing experimental studies that are too small to have adequate power to detect the hypothesized effect. In these studies, too few animals are included to demonstrate a statistically significant effect even when in reality a difference exists. This effect is often quoted as "absence of evidence is not evidence of absence" (Whitley and Ball, 2002b). On the other hand, well-designed studies that do not demonstrate a statistically significant treatment effect (so called *null studies*) are not less valid. These studies are clinically quite important, as they show that a particular procedure may not be indicated, preventing the implementation of potentially harmful procedures.

10.2.10 Materials, Consumables, and Equipment

The instruments, materials, and equipment necessary for horizontal ridge augmentation procedures may be divided into surgical and laboratorial. For the surgical instrumentarium, they should ideally represent instruments utilized in daily clinical practice. There are a vast variety of surgical instruments and materials of different brands, quality, and cost. Personal choice certainly has an important role in selecting instruments and materials, but this choice should not compromise the final results of the surgery. The instruments must be sterile, and the operating room and the animal should be covered with sterile drapes in the same manner as when performing surgery on a human being. To avoid contamination, all materials (bone filler, barrier membranes, or other) must be kept closed in their appropriate sterile packs until needed on the operating table. Ideally, sutures should be nonresorbable and preferably with low plaque retention. More details concerning specific instruments for the surgical part of the experiment or histologic processing and evaluation can be found in Sections 10.2.6 and 10.2.8, respectively.

References

1. Aerssens J, Boonen S, Lowet G, Dequeker J (1998). Interspecies differences in bone composition, density, and quality: potential implications for *in vivo* bone research. *Endocrinology* 139:663–670.
2. Aida T, Yoshioka I, Tominaga K, Fukuda J (2003). Effects of latency period in a rabbit mandibular distraction osteogenesis. *Int J Oral Maxillofac Surg* 32:54–62.
3. Araújo MG, Sonohara M, Hayacibara R, Cardaropoli G, Lindhe J (2003). Lateral ridge augmentation by the use of grafts comprised of autologous bone or a biomaterial. An experiment in the dog. *J Clin Peridontol* 29:1122–1131.
4. Baumgardner KR (1997). A review of key research design and statistical analysis issues. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 84:550–556.
5. Bornstein MM, Bosshardt DD, Buser D (2007). Effect of two different bioresorbable collagen membranes on guided bone regeneration. A comparative histomorphometric study in the dog mandible. *J Periodontol* 78:1943–1953.
6. Bornstein MM, Valderrama P, Jones AA, Wilson TG, Seibl R, Cochran DL (2008). Bone apposition around two different sand-blasted and acid-etched titanium implant surfaces. A histomorphometric study in canine mandibles. *Clin Oral Implants Res* 19:233–241.
7. Bornstein MM, Heynen G, Bosshardt DD, Buser D (2009). Effect of two bioabsorbable barrier membranes on bone regeneration of standardized defects in calvarial bone. A comparative histomorphometric study in pigs. *J Periodontol* 80:1289–1299.
8. Boyde A, Jones SJ (1983). Backscattered electron imaging of dental tissues. *Anat Embryol (Berlin)* 168: 211–226.
9. Boyde A, Jones SJ, Aerssens J, Dequeker J (1995). Mineral density quantitation of the human cortical iliac crest by backscattered electron image analysis: Variations with age, sex, and degree of osteoarthritis. *Bone* 16:619–627.
10. Busenlechner D, Kantor M, Tangl S, Tepper G, Zechner W, Haas R *et al.* (2005). Alveolar ridge augmentation with a prototype trilayer membrane and various bone grafts: a histomorphometric study in baboons. *Clin Oral Implants Res* 16:220–227.
11. Buser D, Schenk RK, Steinemann S, Fiorellini J, Fox C, Stich H (1991). Influence of surface characteristics on bone integration of titanium implants. A histometric study in miniature pigs. *J Biomed Mater Res* 25: 889–902.
12. Buser D, Hoffmann B, Bernard JP, Lussi A, Mettler D, Schenk RK (1998). Evaluation of filling materials in membrane-protected bone defects. A comparative histomorphometric study in the mandible of miniature pigs. *Clin Oral Implants Res* 9:137–150.
13. Buser D, Nydegger T, Oxland T, Cochran DL, Schenk RK, Hirt HP *et al.* (1999). Interface shear strength of titanium implants with a sandblasted and acid-etched surface: a biomechanical study in the maxilla of miniature pigs. *J Biomed Mater Res* 45:75–83.

14. Buser D, Broggini N, Wieland M, Schenk RK, Denzer AJ, Cochran DL *et al.* (2004). Enhanced bone apposition to a chemically modified SLA titanium surface. *J Dent Res* 83:529–533.
15. Chiapasco M, Zaniboni M (2009). Clinical outcomes of GBR procedures to correct peri-implant dehiscences and fenestrations: a systematic review. *Clin Oral Implants Res* 20(Suppl 4):113–123.
16. Chikazu D, Tomizuka K, Ogasawara T, Saijo H, Koizumi T, Mori Y *et al.* (2007). Cyclooxygenase-2 activity is essential for the osseointegration of dental implants. *Int J Oral Maxillofac Surg* 36:441–446.
17. de Kok IJ, Peter SJ, Archambault M, van den Bos C, Kadiyala S, Aukhil I *et al.* (2003). Investigation of allogeneic mesenchymal stem cell-based alveolar bone formation: preliminary findings. *Clin Oral Implants Res* 14:481–489.
18. Dereka XE, Markopoulou CE, Mamalis A, Pepelassi E, Vrotsos IA (2006). Time- and dose-dependent mitogenic effect of basic fibroblast growth factor combined with different bone graft materials: an *in vitro* study. *Clin Oral Implants Res* 17:554–559.
19. Donos N, Kostopoulos L, Karring T (2002). Alveolar ridge augmentation using a resorbable copolymer membrane and autogenous bone grafts. An experimental study in the rat. *Clin Oral Implants Res* 13:203–213.
20. Egermann M, Goldhahn J, Schneider E (2005). Animal models for fracture treatment in osteoporosis. *Osteoporos Int* 16(Suppl 2):S129–S138.
21. Fonseca RJ, Nelson JF, Clark PJ, Frost DE, Olson RA (1983). Revascularization and healing of onlay particulate allogeneic bone grafts in primates. *J Oral Maxillofac Surg* 41:153–162.
22. Hanfelt JJ (1997). Statistical approaches to experimental design and data analysis of *in vivo* studies. *Breast Cancer Res Treat* 46:279–302.
23. Hanisch O, Sorensen RG, Kinoshita A, Spiekermann H, Wozney JM, Wikesjö UM (2003). Effect of recombinant human bone morphogenetic protein-2 in dehiscence defects with non-submerged immediate implants: an experimental study in Cynomolgus monkeys. *J Peridontol* 74:648–657.
24. Hazzard DG, Bronson RT, McClearn GE, Strong R (1992) Selection of an appropriate animal model to study aging processes with special emphasis on the use of rat strains. *J Gerontol* 47:B63–B64.
25. Hwang K, Schmitt JM, Hollinger JO (2000). Interface between titanium miniplate/screw and human calvaria. *J Craniofac Surg* 11:184–188.
26. Jensen SS, Terheyden H (2009). Bone augmentation procedures in localized defects in the alveolar ridge: clinical results with different bone grafts and bone-substitute materials. *Int J Oral Maxillofac Implants* 24(Suppl):218–236.
27. Jensen SS, Broggini N, Hjørting-Hansen E, Schenk R, Buser D (2006). Bone healing and graft resorption of autograft, anorganic bovine bone and beta-tricalcium phosphate. A histologic and histomorphometric study in the mandibles of minipigs. *Clin Oral Implants Res* 17:237–243.
28. Jensen SS, Bornstein MM, Dard M, Bosshardt DD, Buser D (2009). Comparative study of biphasic calcium phosphates with different HA/TCP ratios in mandibular bone defects. A long-term histomorphometric study in minipigs. *J Biomed Mater Res B Appl Biomater* 90B:171–181.
29. Karnowsky MJ (1965). A formaldehyde-glutaraldehyde fixation of high osmolarity for use in electron microscopy. *J Cell Biol* 27:137A–138A.
30. Katsaros C, Zissis A, Bresin A, Kiliaridis S (2006). Functional influence on sutural bone apposition in the growing rat. *Am J Orthod Dentofac Orthop* 129: 352–357.
31. Kon K, Shiota M, Ozeki M, Yamashita Y, Kasugai S (2009). Bone augmentation ability of autogenous bone graft particles with different sizes: a histological and micro-computed tomography study. *Clin Oral Implants Res* 20:1240–1246.
32. Kostopoulos L, Karring T (1994). Augmentation of the rat mandible using guided tissue regeneration. *Clin Oral Implants Res* 5:75–82.
33. Liebschner MA (2004). Biomechanical considerations of animal models used in tissue engineering of bone. *Biomaterials* 25:1697–1714.
34. Mai R, Reinstorf A, Pilling E, Hlawitschka M, Jung R, Gelinsky M *et al.* (2008). Histologic study of incorporation and resorption of a bone cement-collagen composite: an *in vivo* study in the minipig. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 105:e9–e14.
35. Maréchal M, Luyten F, Nijs J, Postnov A, Schepers E, van Steenberghe D (2005). Histomorphometry and micro-computed tomography of bone augmentation under a titanium membrane. *Clin Oral Implants Res* 16:708–714.
36. Miranda DA, Blumenthal NM, Sorensen RG, Wozney JM, Wikesjö UM (2005). Evaluation of recombinant human bone morphogenetic protein-2 on the repair of alveolar ridge defects in baboons. *J Peridontol* 76:210–220.
37. Muschler GF, Raut VP, Patterson TE, Wenke JC, Hollinger JO (2010). The design and use of animal models for translational research in bone tissue engineering and regenerative medicine. *Tissue Eng Part B Rev* 16:123–145.
38. Nanci A, Zalzal S, Smith CE (1990). Routine use of backscattered electron imaging to visualize cytochemical and autoradiographic reactions in semi-thin plastic sections. *J Histochem Cytochem* 38:403–414.
39. O'Loughlin PF, Morr S, Bogunovic L, Kim AD, Park B, Lane JM (2008). Selection and development of preclinical models in fracture-healing research. *J Bone Joint Surg Am* 90(Suppl 1):79–84.
40. Olsen ML, Aaboe M, Hjørting-Hansen E, Hansen AK (2004). Problems related to an intraoral approach for experimental surgery on minipigs. *Clin Oral Implants Res* 15:333–338.
41. Orsini G, Scarano A, Piattelli M, Piccirilli M, Caputi S, Piattelli A (2006). Histologic and ultrastructural analysis of regenerated bone in maxillary sinus augmentation using a porcine bone-derived biomaterial. *J Periodontol* 7:1984–1990.

42. Pearce AI, Richards RG, Milz S, Schneider E, Pearce SG (2007). Animal models for implant biomaterial research in bone: a review. *Eur Cells Mater* 13:1–10.
43. Reinwald S, Burr D (2008). Review of nonprimate, large animal models for osteoporosis research. *J Bone Miner Res* 23:1353–1368.
44. Rocchietta I, Dellavia C, Nevins M, Simion M (2007). Bone regenerated via rhPDGF-bB and a deproteinized bovine bone matrix: backscattered electron microscopic element analysis. *Int J Periodontics Restorative Dent* 27:539–545.
45. Saffarzadeh A, Gauthier O, Bilban M, Bagot D'Arc M, Daculsi G (2009). Comparison of two bone substitute biomaterials consisting of a mixture of fibrin sealant (Tisseel) and MBCP (TricOs) with an autograft in sinus lift surgery in sheep. *Clin Oral Implants Res* 20: 1133–1139.
46. Sawai T, Niimi A, Takahashi H, Ueda M (1996). Histologic study of the effect of hyperbaric oxygen therapy on autogenous free bone grafts. *J Oral Maxillofac Surg* 54:975–981.
47. Schenk RK, Olah AJ, Hermann W (1984). Preparation of calcified tissues for light microscopy. In: Methods of calcified tissue preparation, 1st ed. Dickson GR, editor. Amsterdam: Elsevier, pp. 1–56.
48. Schimandle JH, Boden SD (1994). Spine update. The use of animal models to study spinal fusion. *Spine* 19:1998–2006.
49. Schwarz F, Herten M, Ferrari D, Wieland M, Schmitz L, Engelhardt E *et al.* (2007). Guided bone regeneration at dehiscence-type defects using biphasic hydroxyapatite + beta tricalcium phosphate (Bone Ceramic) or a collagen-coated natural bone mineral (BioOss Collagen): an immunohistochemical study in dogs. *Int J Oral Maxillofac Surg* 36:1198–1206.
50. Schwarz F, Rothamel D, Herten M, Wüstefeld M, Sager M, Ferrari D *et al.* (2008a). Immunohistochemical characterization of guided bone regeneration at a dehiscence-type defect using different barrier membranes: an experimental study in dogs. *Clin Oral Implants Res* 19:402–415.
51. Schwarz F, Rothamel D, Herten M, Ferrari D, Sager M, Becker J (2008b). Lateral ridge augmentation using particulated or block bone substitutes biocoated with rhGDF-5 and rhBMP-2: an immunohistochemical study in dogs. *Clin Oral Implants Res* 19:642–652.
52. Schwarz F, Sager M, Ferrari D, Mihatovic I, Becker J (2009). Influence of recombinant human platelet-derived growth factor on lateral ridge augmentation using biphasic calcium phosphate and guided bone regeneration: a histomorphometric study in dogs. *J Periodontol* 80:1315–1323.
53. Stratton IM, Neil A (2005). How to ensure your paper is rejected by the statistical reviewer. *Diabet Med* 22: 371–373.
54. Traini T, Degidi M, Sammons R, Stanley P, Piattelli A (2008). Histologic and elemental microanalytical study of anorganic bovine bone substitution following sinus floor augmentation in humans. *J Periodontol* 79:1232–1240.
55. Tung K, Fujita H, Yamashita Y, Takagi Y (2006). Effect of turpentine-induced fever during the enamel formation of rat incisor. *Arch Oral Biol* 51:464–470.
56. von Arx T, Cochran DL, Hermann JS, Schenk RK, Buser D (2001a). Lateral ridge augmentation using different bone fillers and barrier membrane application. A histologic and histomorphometric pilot study in the canine mandible. *Clin Oral Implants Res* 12:260–269.
57. von Arx T, Cochran DL, Hermann JS, Schenk RK, Higginbottom FL, Buser D (2001b). Lateral ridge augmentation and implant placement: an experimental study evaluating implant osseointegration in different augmentation materials in the canine mandible. *Int J Oral Maxillofac Implants* 16:343–354.
58. von Arx T, Cochran DL, Schenk RK, Buser D (2002). Evaluation of a prototype trilayer membrane (PTLM) for lateral ridge augmentation: an experimental study in the canine mandible. *J Oral Maxillofac Surg* 31:190–199.
59. Wang X, Mabrey JD, Agrawal CM (1998). An interspecies comparison of bone fracture properties. *Biomed Mater Eng* 8:1–9.
60. Whitley E, Ball J (2002a). Statistics review 3: hypothesis testing and P values. *Crit Care* 6:222–225.
61. Whitley E, Ball J (2002b). Statistics review 4: sample size calculations. *Crit Care* 6:335–341.
62. Ylinen P, Raekallio M, Toivonen T, Vihtonen K, Vainionpää S (1991). Preliminary study of porous hydroxylapatite particle containment with a curved biodegradable implant in the sheep mandible. *J Oral Maxillofac Surg* 49:1191–1197.